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MINIREVIEW

Posttranslational Modifications of Proteins in the Pathobiology of Medically Relevant Fungi

Michelle D. Leach and Alistair J. P. Brown

School of Medical Sciences, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, United Kingdom

Posttranslational modifications of proteins drive a wide variety of cellular processes in eukaryotes, regulating cell growth and division as well as adaptive and developmental processes. With regard to the fungal kingdom, most information about posttranslational modifications has been generated through studies of the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, where, for example, the roles of protein phosphorylation, glycosylation, acetylation, ubiquitination, sumoylation, and neddylation have been dissected. More recently, information has begun to emerge for the medically important fungal pathogens *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, highlighting the relevance of posttranslational modifications for virulence. We review the available literature on protein modifications in fungal pathogens, focusing in particular upon the reversible peptide modifications sumoylation, ubiquitination, and neddylation.

The biological exploration of fungal pathogens, many of which lack an exploitable sexual cycle, has often focused upon protein/gene expression levels. Indeed, changes in protein (or transcript) abundance have provided valuable clues about the cellular processes that contribute to fungal pathogenesis. However, measurements of protein (or transcript) abundances alone are not sufficient to understand the regulation of such processes, because the activities of many eukaryotic proteins are modulated at posttranslational levels.

Posttranslational modifications are covalent processing events that often change the properties of a protein, for example, by proteolytic cleavage or by the addition of a modifying group to one or more amino acid residues (Table 1). Some posttranslational modifications make major structural contributions to their target proteins. For example, the glycosylation of cell wall mannoproteins fundamentally influences the shape of the fungal cell surface and hence the interactions of fungal pathogens with their hosts (86, 91, 100). Other regulatory modifications determine the activity state of specific proteins, their stability, their localization, and/or their interactions with other proteins. In signal transduction pathways, for example, protein kinase cascades are activated and inactivated by the reversible addition and removal of phosphate groups to specific residues in signaling proteins (22). Moreover, the posttranslational marking of cyclins by ubiquitination targets them for destruction during defined phases of the cell cycle (170).

For cellular processes to be regulated with requisite accuracy, posttranslational modification must be executed with exquisite specificity with regard to the choice of protein target, the selection of the residues to be modified, and the nature of the modifying group. Posttranslational modification mechanisms and their regulatory significance have been studied extensively in the model yeast *Saccharomyces cerevisiae*, and more recent data have highlighted the contributions of specific regulatory modifications to fungal pathogenicity. The main focus of this review is the reversible peptide modifications ubiquitination, neddylation, and sumoylation, whose contribution to fungal pathogenicity has been a topic of recent study. To place these modifications in context, we begin with a brief overview of posttranslational modifications and their impact upon fungal virulence (Table 1).

POSTTRANSLATIONAL MODIFICATIONS AND VIRULENCE

The fungal cell wall is the first point of contact between the host and pathogen and therefore plays an important role in the adherence to and colonization of host tissues and in host recognition. Numerous cell surface proteins are posttranslationally modified in fungi, and disruption of these modifications can significantly attenuate virulence. For example, cell wall and secreted mannoproteins are highly glycosylated in *S. cerevisiae* and *Candida albicans* (6, 113) (Table 1). The importance of *N*-glycosylation for fungal pathogenesis has been confirmed, for example, by disruption of the α1,6-mannosyltransferase Och1 (6). Och1 initiates a distinct branch on the *N*-glycan core, providing the platform for subsequent addition of the extensively mannosylated outer chains. *C. albicans och1* mutants display major cell wall defects and attenuated virulence in a murine model of infection (6).

O-Mannosylation is initiated in the endoplasmic reticulum (ER) by the family of Dol-P-Man:protein *O*-mannosyltransferases (PMTs) (100). The characterization of *pmt* mutants has clearly demonstrated the essentiality of protein *O*-mannosylation in fungi. Tanner and coworkers showed that particular combinations of triple *pmt* mutants are lethal in *S. cerevisiae* (e.g., *pmt1 pmt2 pmt4* or *pmt2 pmt3 pmt4*) (44). In pathogenic fungi, *O*-linked mannosylation is required for virulence. Loss of Pmt1, Pmt2, Pmt4, Pmt5, or Pmt6 attenuates the virulence of *C. albicans* (136, 148, 165, 166). Furthermore, loss of *PMT4* or *PMT1* in *Cryptococcus neoformans* attenuates the virulence of this pathogen both in murine models of disseminated cryptococcal virulence (124) and in murine inhalation models of cryptococcosis (178). *Aspergillus* species also retain a highly conserved *PMT* gene family, and single *pmt2* and double *pmt1 pmt4* deletions are lethal in *Aspergillus fumigatus* (115). The importance of *N*- and *O*-glycosylation in pathogenic fungi can be largely attributed to their key roles in the

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Address correspondence to Michelle D. Leach, michelle.leach@abdn.ac.uk.

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TABLE 1 Processes regulated by posttranslational modifications

Posttranslational modification	Cellular process (reference)	Exemplar target proteins (reference[s])	Mechanisms (reference[s])
Phosphorylation	Signaling	MAP kinases (112, 143) Two-component regulators (80, 186) Transcription factors (7, 118)	Addition of a phosphate group(s) to specific motifs often consisting of a few key residues surrounding the target amino acid (171)
Glycosylation	Cell surface	Cell wall proteins (6, 76)	*N*-Glycosylation: attachment of an inner core unit to asparagine residues in target proteins via an amide linkage in the endoplasmic reticulum (43)
	Host defense	Mannoproteins (6, 113)	*O*-Linked glycosylation: attachment of shorter linear glycans via either linkages to serine or threonine residues of target proteins (167)
Acetylation	Gene regulation (93)	Histones (81)	Acetylation: covalent addition of the acetyl chemical group to lysine residues in histones and other proteins, catalyzed by histone acetyltransferases (81)
	DNA replication (48)	Basal transcription factors (64)	*N*-Acetylation: protein N termini can be modified by acetylation of the free α-amino group by *N*-α-acetyl transferases (133)
	DNA repair (48)	Site-specific DNA binding factors (149) Architectural DNA binding factors (5, 15)	
Methylation	Development and differentiation	Ribosomal proteins (134) Translation factors (134) Histones (104)	Addition of methyl groups to amino acid residues such as arginine (134)
Proteolysis	Cell-cell communication Transcriptional regulation	Transcription factors (96) Pheromones (9, 83)	Process of breaking peptide bonds between amino acids in proteins, carried out by peptidases and proteases (1)
Disulfide bond formation	Protein structure Protein function	Cell wall proteins (135) Transcription factors (123)	Disulfide bonds are formed between the sulfur atoms of pairs of cysteine residues within or across proteins, and is catalyzed by protein disulfide isomerase (177)
GPI anchors	Signal transduction Cell-cell interaction (142) Host defense	Cell wall proteins (16, 42) Plasma membrane proteins (16)	GPI anchoring is a mechanism to tether proteins to the cell surface via an amide linkage; it has been extensively reviewed in yeast (41, 126)
Palmitoylation	Membrane interactions Protein-protein interactions Enzyme activity	Cytoplasmic proteins (157) G-proteins (157, 168)	Palmitoylation is the thioester linkage of long-chain fatty acids to cysteine residues in proteins (27, 157)
Farnesylation	Signaling Protein-protein interactions	G-proteins (51) Pheromones (3)	Farnesylation occurs by formation of cysteine thioethers with farnesyl at or near the C-terminus (17, 155)
Ubiquitination	Cell cycle progression Gene transcription DNA repair Adaptation	Translation factors (162) Metabolic proteins (107) Cell cycle (162) Stress proteins (107)	Ubiquitination involves the modification of target proteins through the covalent attachment of ubiquitin via an isopeptide bond with target lysine residues of a substrate protein (55, 57)
Neddylation	Cell growth Development	Cullins (85) Ribosomal proteins (183)	Neddylation involves the covalent addition of the NEDD8 polypeptide to target proteins via an isopeptide linkage between the C-terminal glycine of NEDD8 and a lysine on the target protein (138)
Sumoylation	Transcriptional regulation Cell cycle Stress responses	Transcription factors (52) Heat shock proteins (189) Histones (179)	SUMO is covalently attached through an isopeptide bond to the ε-amino group of target lysine residues in specific protein substrates (53)

construction and maintenance of a robust cell wall, an essential structure in fungi.

Glycosylphosphatidylinositol (GPI) anchoring of proteins on the cell surface also plays a role in fungal virulence (Table 1). For example, the GPI-anchored proteins Phr1, Phr2, and Utr2 are involved in *C. albicans* cell wall biosynthesis and modeling (142). Also, Sod5 combats macrophage-induced oxidative stress (38), the phospholipase B Plb5 promotes the colonization of organs *in*

vivo (164), and ALS protein family members and Hwp1 enhance adherence to host tissues (60, 154).

Stress responses and environmental adaptation are of particular significance for fungal pathogens, as they must mount effective responses to counteract the defenses of their host and adapt to available nutrients in diverse host niches. This adaptation is frequently achieved through posttranslational modifications of signaling proteins (Table 1). For example, Ras GTPases are highly conserved signaling proteins that play central roles in key physiological processes, such as growth, morphology, and survival. Ras1 in *C. albicans* signals through the cyclic AMP (cAMP) or Cek1 mitogen-activated protein (MAP) kinase signaling cascades (30) initiating hyphal growth, a key virulence trait of *C. albicans*. Ras1 localization to the plasma membrane, which is critical for activation, is controlled by farnesylation and palmitoylation in *C. albicans* (132). In *C. neoformans*, palmitoylation is required for Ras1 localization, which is necessary for morphogenesis (120). Additionally, protein phosphorylation, a ubiquitous and reversible modification that is crucial for the regulation of multiple cellular events (153), regulates MAP kinase and two-component signaling cascades in fungal pathogens (80, 186). Also, protein phosphorylation via cAMP-protein kinase A signaling contributes to fungal virulence through regulation of yeast-hypha morphogenesis and stress resistance in *C. albicans* (13, 14, 45) and mating, capsule formation, and melanin production in *C. neoformans* (2). Not surprisingly, protein phosphorylation has been studied extensively, and its role in yeast biology and fungal pathogenicity has been reviewed elsewhere (7, 112, 143).

Signaling pathways often culminate in the phosphorylation of specific transcription factors that drive the changes in gene expression patterns that underpin the corresponding adaptive or developmental process. For example, the heat shock transcription factor Hsf1, which controls thermal adaptation in *C. albicans* and other eukaryotic cells, is phosphorylated in response to heat shock (118). Hsf1 phosphorylation is required for adaptation to thermal insults and also for the virulence of *C. albicans* (119). However, the activity of transcription factors can be modified by other types of posttranslational modification. For instance, the activity of the pH-responsive transcription factor Rim101, which is necessary for *in vivo* pathogenesis in *C. albicans* (25), is modulated by the proteolytic removal of 100 carboxy-terminal residues (96).

Accurate genome organization, gene regulation, DNA replication, and DNA repair are essential for growth and pathogenicity. These processes are frequently controlled by histone acetylation, a posttranslational modification that plays a major role in the modulation of high-order chromatin structures. In the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, newly synthesized histone H3 molecules are heavily acetylated on lysine 56 (H3K56) (72, 106, 181). This occurs through the fungus-specific histone acetyltransferase (HAT) enzyme, Rtt109, and the histone chaperone Asf1 (28, 50, 139, 169). Loss of Rtt109 or Asf1 in *S. cerevisiae* causes delayed cell cycle progression (28) and spontaneous DNA damage (32, 106), and blocked acetylation at H3K56 results in similar phenotypes (106, 139). Therefore, the acetylation of H3K56 appears to be a particularly important posttranslational modification for fungal growth.

Recent studies have shown that Rtt109 (and presumably H3K56 acetylation) is required for *C. albicans* pathogenicity. *C. albicans rtt109* mutants are relatively sensitive to genotoxic agents, highly susceptible to macrophage killing, and less virulent than the wild type in a mouse model of systemic infection (102). Interestingly, the increased sensitivity to macrophages is dependent on the host's ability to produce reactive oxygen species (ROS), indicating that acetylation also contributes to stress adaptation. Histone (de)acetylation also contributes to morphogenetic regulation and phenotypic switching in *C. albicans*, both of which promote virulence (56, 159).

Histone acetylation has also been shown to contribute to the pathogenicity of *C. neoformans*. In *S. cerevisiae* the histone acetyltransferase Gcn5 is involved in the regulation of transcriptional responses to various environmental stresses, including high temperature, osmotic stress, nutrient deprivation, and oxidative damage (61). Similarly, *C. neoformans gcn5* mutants show defects in growth at high temperatures, sensitivity to oxidative stress and FK506 (a calcineurin inhibitor), and defects in capsule attachment to the cell surface (125). Stress adaptation and capsule formation promote *C. neoformans* pathogenesis, and therefore it is not surprising that the *gcn5* mutants are avirulent in animal models of cryptococcosis (125).

Protein methylation also contributes to a range of cellular processes, including protein transport, transcriptional regulation, and signaling (Table 1). For example, protein arginine methylation by Hmt1 has been shown to promote the nuclear export of target proteins in *C. albicans* (108). However, the impact of protein methylation upon fungal pathogenicity remains to be tested.

The reversible addition to proteins of specific peptide moieties, such as ubiquitin, NEDD, and SUMO, has been shown to influence fungal virulence (Table 1). These posttranslational modifications are discussed in the next sections.

REVERSIBLE PEPTIDE MODIFICATIONS

Ubiquitination. Ubiquitination involves the posttranslational modification of target proteins through the covalent attachment of ubiquitin (Ub), a highly conserved 76-amino-acid protein that has the unusual property of forming a stable chemical bond with other proteins. The carboxyl group of the carboxy-terminal glycine of ubiquitin forms an isopeptide bond with the ε-amino group of target lysine residues, or occasionally with the amino group at the amino terminus of a substrate protein (55, 57). Ubiquitin conjugation is achieved via a three-step reaction catalyzed by three enzymes: E1, a ubiquitin-activating enzyme; E2, a ubiquitin-conjugating enzyme; and E3, a ubiquitin ligase. Briefly, the activation of ubiquitin by E1 is followed by the conjugation of ubiquitin to an E2 enzyme. Finally, ubiquitin is transferred to the protein substrate or target by an E3 ubiquitin ligase (184; see reference 166 for a review). Ubiquitination is reversible, like the process of (de)phosphorylation. The cleavage of ubiquitin from substrates is carried out by specific deubiquitinating enzymes (58).

Ubiquitination can modulate the activity of target proteins in various ways. These include the proteasome-mediated degradation of some target proteins and the stabilization of others through deubiquitination (110). Ubiquitination can also influence the activity of some proteins, or their cellular localization. Ubiquitin modifications take the form of monoubiquitin (attachment of a single ubiquitin moiety), multiple monoubiquitination (modification of several target lysines with a single ubiquitin), or polyubiquitination (attachment of four or more ubiquitin moieties) (175). The different types of ubiquitin conjugates control different cellular processes. Polyubiquitination generally targets the substrates for degradation, whereas the addition of fewer ubiq-

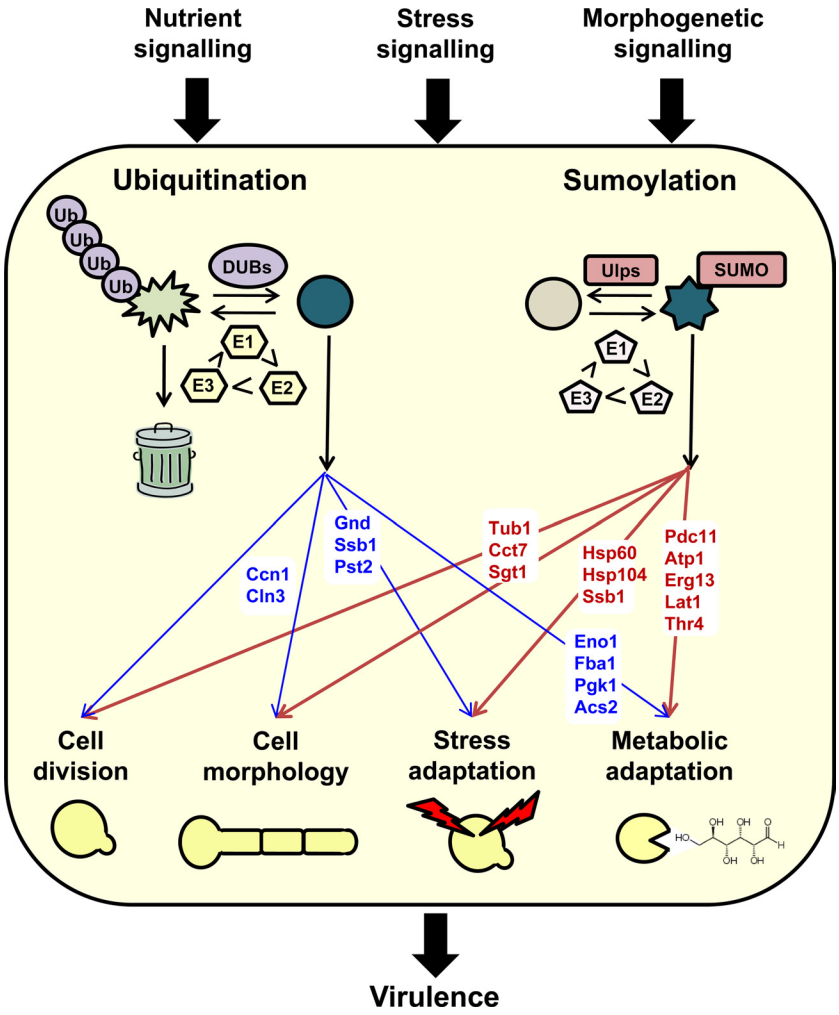

FIG 1 Roles of ubiquitination and sumoylation. Polyubiquitinated proteins are targeted to the proteasome for degradation via E1, E2, and E3 enzymes. Dubs (deubiquitination enzymes) reverse this process. Ubiquitin targets a number of proteins involved in different cellular processes (blue lines). Sumoylation contributes to similar processes (red lines) but modifies proteins by attaching SUMO via E1, E2, and E3 enzymes, and this modification generally activates a protein. Sumoylation is reversible by ULP proteases. These modifications are often triggered by specific environmental stimuli, and the cellular processes they regulate contribute to fungal pathogenicity.

uitin molecules can alter the substrate protein function or target the substrate protein to the endosome (185). Proteins involved in many cellular processes are targets for ubiquitination, in many cases their ubiquitination acting as a signal for recognition by an ubiquitin-binding protein. Therefore, ubiquitination contributes to the regulation of numerous cellular processes, including cell cycle progression, gene transcription, DNA repair, and inflammation (49, 55, 116, 131).

In *S. cerevisiae*, ubiquitin is encoded by a multigene family of natural gene fusions (*UBI1*, *UBI2*, *UBI3*, and *UBI4*). *UBI1*, *UBI2*, and *UBI3* encode hybrid proteins in which ubiquitin is fused to unrelated amino acid sequences (128). *UBI1* and *UBI2* encode identical 52-residue polypeptide tails, which are components of the large ribosomal subunit. In contrast, *UBI3* encodes a different 76-residue tail that corresponds to the S34 protein of the small ribosomal subunit (33, 128). The fourth ubiquitin gene in *S. cerevisiae*, *UBI4*, encodes a polyubiquitin precursor protein comprising five tandem repeats of ubiquitin in a spacerless head-to-tail arrangement (128).

All four ubiquitin genes are expressed in exponentially growing *S. cerevisiae* cells, but the expression of *UBI1* and *UBI2* is repressed in stationary-phase cells. Mutants lacking the ubiquitin-hybrid genes display slow-growth phenotypes, particularly in the case of the *ubi3* deletion. *UBI1* and *UBI2* encode functionally redundant proteins that execute an essential function, as a *ubi1 ubi2* double mutant is not viable (33). The *UBI4* gene is strongly induced by starvation, high temperatures, and oxidative stress (18, 35), and overexpression of *UBI4* restores heat shock sensitivity in the absence of the enzyme serine palmitoyltransferase, required for resistance to heat shock (40). Therefore, *UBI4* is thought to provide ubiquitin under stress conditions (18, 34, 35, 128). The roles of ubiquitin in fungal pathogens have been less well characterized than those in model yeasts, but recent data strongly implicate polyubiquitin in growth, metabolism, and stress responses (Fig. 1).

(i) Ubiquitination and stress. Two ubiquitin-encoding genes have been identified in *C. albicans*, *C. neoformans*, and *Aspergillus nidulans*. These are *UBI3* and *UBI4* in *C. albicans* and *UBI1* and

UBI4 in *C. neoformans* and *A. nidulans*. As yet, these *C. neoformans* genes have not been characterized in any detail: they have been isolated (158), but they have yet to be dissected functionally. Nevertheless, the involvement of the ubiquitination system in stress adaptation has been examined in *C. neoformans*, with two ubiquitin-conjugating genes, *UBC6-2* and *UBC8*, being implicated in oxidative stress responses (77). Furthermore, the expression of the F-box protein Fbp1 is regulated by glucose in *C. neoformans*, highlighting a potential role for this SCF (for "Skp1/Cdc53/F-box protein") complex (and hence ubiquitination) in glucose sensing by this pathogen. (F-box proteins are exchangeable subunits that promote the capacity of SCF to specifically target protein substrates for ubiquitination and degradation.) Significantly, a *C. neoformans fbp1* mutant is unable to generate spores and displays attenuated virulence in a murine intranasal-inhalation model, highlighting the key role of the SCF E3 ubiquitin ligase-mediated pathway in these processes (99).

In the model filamentous fungus *A. nidulans*, the regulation of the polyubiquitin-encoding *ubi1* and *ubi4* transcripts has been characterized, with specific focus on stress. Both genes are induced upon heat stress, but only the *ubi1* gene is induced by peroxide stress (121), suggesting that these ubiquitin genes play differential roles in stress adaptation. Similarly, recent work on *C. albicans* has highlighted differential roles for the ubiquitin-encoding genes *UBI3* and *UBI4* in stress adaptation. The polyubiquitin gene *UBI4* is induced in response to numerous stress responses, including heat, peroxide, and cell wall stresses (88). This contrasts with a ubiquitin hybrid protein, Ubi3, which is involved in ribosome biogenesis and which is coordinately regulated with components of the translational apparatus (145, 147).

A number of stress-related proteins have been identified as ubiquitination targets under stress conditions in *C. albicans*. These include Pst2 (an oxidative-stress response protein), which is ubiquitinated in response to peroxide stress, and Ssb1 (a member of the Hsp70 chaperone family), which is ubiquitinated in response to both peroxide and heat stress (88). During heat stress, chaperone proteins are upregulated to prevent accumulation of aggregated proteins and to promote the refolding of unfolded proteins (47, 130). Increasing ubiquitin-dependent degradation can suppress this normally essential requirement for heat shock protein induction (40). This would seem to imply that an essential aspect of stress adaptation is the removal of misfolded proteins, either by refolding or through ubiquitin-mediated degradation.

Heat shock proteins such as Hsp70 and Hsp90 have been implicated in the maintenance of the ubiquitin-proteasome system, specifically, the 26S proteasome in *S. cerevisiae* (62, 63). Furthermore, Ssa1 (another Hsp70 family member) has been linked to protein quality control, directing cytoplasmically mislocalized proteins for proteasomal degradation (109, 129). More recently, a role for Ssa1 in regulating ubiquitination and degradation of correctly folded gluconeogenic enzymes was elucidated, demonstrating that the Hsp70 chaperone machinery is involved in regulatory degradation of metabolic enzymes (70). Indeed, a positive feedback loop may exist, whereby ubiquitination of stress-related proteins might activate them to work with the polyubiquitin pathway in the degradation of aggregated proteins, whose inherent toxicity would be detrimental to the cell. A second hypothesis may be that chaperone proteins are merely targeted for degradation after an initial upregulation during temperature stress. These ideas are not mutually exclusive. It would be interesting to test whether such a feedback loop, involving the regulation of ubiquitination by ubiquitinated proteins, exists in fungi.

(ii) Ubiquitination and metabolism. Both metabolic diversity and efficiency are required for microorganisms to successfully exploit the dynamically changing microenvironments they occupy. This flexibility can be achieved through gene regulation but also through posttranslational modifications such as ubiquitination. Indeed, ubiquitination has been shown to be required for the survival of *S. cerevisiae* under starvation conditions (176). Also, a number of specific proteins are degraded via the proteasome during metabolic transitions, for example, when cells switch from gluconeogenic to glycolytic metabolism. Fructose-1,6-bisphosphatase (FBPase) (150, 151), Std1 (74, 114), and the regulatory protein Mth1 (37) are rapidly degraded following glucose addition to *S. cerevisiae* cells. Interestingly, when glucose is abundant, Mth1 is targeted for proteasomal degradation by Grr1 (an F-box protein) (36, 37). What is more, cells lacking Grr1 have several metabolic defects, including sensitivity to nitrogen starvation (36).

A number of metabolic proteins have been identified as ubiquitination targets in *C. albicans*, including proteins involved in glycolysis and/or gluconeogenesis (Eno1, Fba1, and Pgk1), the pentose phosphate pathway (Tkl1), fatty acid metabolism (Pdx3), acetate utilization (Asc2), and glycerol synthesis (Gpd2) (88). Furthermore, a lack of polyubiquitin (Ubi4) reduces the ability of *C. albicans* to survive under starvation conditions. Strikingly, *C. albicans ubi4* mutants rapidly lost viability when cells were starved for nitrogen in the presence of excess carbon, suggesting that ubiquitin-mediated protein degradation is required for nitrogen recycling under these conditions. Furthermore, specific *C. albicans* proteins are rapidly degraded in response to glucose (173), although the exact mechanisms by which this occurs are yet to be revealed. Interestingly, the ubiquitin ligase gene, *UBC8*, whose orthologue is involved in glucose-accelerated protein degradation in *S. cerevisiae* (140, 152), is conserved in *C. albicans* (122). Furthermore, *UBC8* is the only ubiquitin-related gene that is upregulated at the transcriptional level following glucose exposure in *C. albicans* (144). Therefore, analogous ubiquitin-dependent mechanisms probably contribute to metabolic adaptation in *S. cerevisiae* and *C. albicans* and may exist in other fungal pathogens.

(iii) Ubiquitination, growth, and morphogenesis. Roles for ubiquitination in morphogenesis and other developmental processes have been reported for both *C. albicans* and *A. nidulans*. Interestingly, *A. nidulans* GrrA (which is closely related to *S. cerevisiae* Grr1) plays a role in meiosis and sexual spore formation. Deleting *grrA* inhibits the formation of mature ascospores due to a block in meiosis (79). In *C. albicans*, the deletion of *UBI4* leads to a heterogeneous mixture of yeast and hyphal cells (88, 146) whereby the hyphal cells display an apparent mitotic arrest (88). The exact mechanisms by which this occurs are currently unknown. However, it is well known that cellular morphogenesis is intimately linked with cell cycle regulation in many eukaryotes, particularly in budding yeasts (92). Also, ubiquitin-mediated protein degradation plays a central role in cell cycle control, with cyclin degradation being tightly regulated by ubiquitin-dependent proteolysis (78, 188). The multiprotein ubiquitin ligase SCF plays an important role in cell cycle control by degrading key proteins involved in cell cycle regulation in *S. cerevisiae* and other eukaryotes (20, 78, 188). In *S. cerevisiae*, the F-box protein Cdc4 (SCF^{Cdc4}) is required for degradation of the cyclin-dependent kinase inhibitors Sic1 (117) and Far1 (12), while its orthologue in *C. albicans* has a role in

regulating morphogenesis (4). Additionally, SCF^{Grr1} targets the G_1 cyclins Cln1 and Cln2 for degradation in *S. cerevisiae* (10, 75); similarly, deletion of *GRR1* stabilizes two G_1 cyclins, Ccn1 and Cln3, with the mutant growing constitutively as pseudohyphae (97). Also, the ubiquitin-conjugating (E2) enzyme Rad6 has been shown to downregulate hyphal development in *C. albicans* (90).

Clearly, the ubiquitination pathway contributes to the regulation of diverse cellular processes in fungal pathogens, including cell division and developmental processes as well as stress and metabolic adaptation. Given the fundamental importance of these processes, it is hardly surprising that ubiquitination contributes significantly to fungal virulence (88). However, the exact mechanisms by which ubiquitination regulates these cellular processes and hence fungal pathogenesis needs further dissection.

Sumoylation. Like ubiquitination, sumoylation is a highly conserved, reversible posttranslational modification that involves the conjugation of a polypeptide to target proteins. The SUMO (small ubiquitin-like modifier) polypeptide is expressed in all eukaryotes (29, 39, 54). All yeasts and invertebrates studied to date contain a single SUMO gene, whereas vertebrates contain three SUMO genes, SUMO-1, SUMO-2, and SUMO-3 (68, 71, 103). Plants contain additional SUMO genes, with eight such genes having been found in *Arabidopsis* (82).

SUMO is covalently attached through an isopeptide bond to the ε-amino group of target lysine residues in specific protein substrates. This occurs via a three-step enzymatic pathway that is roughly analogous to the ubiquitination pathway (46, 111). Briefly, after proteolytic maturation, the *S. cerevisiae* SUMO protein, Smt3, is activated in an ATP-dependent fashion by a SUMO-activating enzyme, E1, through the formation of a thioester bond between its carboxy terminus and the E1-activating complex, Uba2/Aos1. Activated SUMO is then transferred to the E2-conjugating enzyme, Ubc9. SUMO is then transferred from Ubc9 to the substrate protein with the assistance of one of the several SUMO protein ligases (Siz1 and Siz2 in *S. cerevisiae*), termed E3s. Ultimately, Ubc9 catalyzes the covalent link between the carboxy-terminal glycine in Smt3 and a lysine residue present in the sumoylation consensus sequence of the target protein.

Ubc9 contributes to substrate recognition and Smt3 ligation (8), whereas E3 ligases play important roles in enhancing substrate identification and specificity (111). Many target lysine residues lie in the short consensus sequence ΨKXE, where Ψ is a large hydrophobic amino acid (generally isoleucine, leucine, or valine), K is the lysine residue that is modified, X is any residue, and E is glutamic acid. Sumoylation is a reversible modification, and removal of SUMO is carried out by enzymes of the Ulp family that specifically cleave at the carboxy terminus of SUMO (94, 95).

In *S. cerevisiae*, the single SUMO gene, *SMT3*, is essential for viability. In the fission yeast, *S. pombe*, cells lacking the SUMO gene *pmt3* are barely viable and have defects in genome maintenance (163). In *S. cerevisiae*, sumoylation regulates a variety of cellular processes, including septin ring formation (66, 67), sister chromatid cohesion (11, 160), DNA repair (65, 172), and transcriptional regulation (156). Unlike polyubiquitination, which generally targets proteins for degradation, sumoylation generally exerts its regulatory effects upon target proteins by modulating protein-protein interactions and protein localization. In some cases, sumoylation antagonizes ubiquitin conjugation, thereby promoting protein stabilization (26, 46). Recent studies have revealed that there is cross talk between sumoylation and ubiquitination, whereby sumoylation can acts as a target for SUMO-directed ubiquitin ligases (137, 161, 182).

Virtually nothing is known about sumoylation in the pathogenic fungi. To date, there are only two studies of sumoylation in *C. albicans* and *A. nidulans*, with no reports for *C. neoformans*. Interestingly, the SUMO gene is not essential in *C. albicans* or *A. nidulans*, but mutants that lack SUMO exhibit impaired growth. In *A. nidulans*, *sumO*Δ cells display reduced conidiation and increased sensitivity to the DNA-damaging agent methyl methanesulfonate (MMS) and to the DNA synthesis inhibitor hydroxyurea (HU) (180). *sumO*Δ cells also display self-sterility, suggesting that sumoylation of key targets is required for the development of viable meiotic progeny.

The inactivation of *SMT3* in *C. albicans* generates a heterogeneous population of slowly growing, enlarged, elongated, pseudohypha-like cells (87). This suggested defects in cell cycle progression, and this was confirmed when DAPI (4′,6′-diamidino-2-phenylindole) staining revealed many multinucleate *C. albicans smt3* cells (87). The possibility that septin sumoylation is required for normal septation might provide an explanation for this phenotype. *S. cerevisiae* mutants with defects in any one of the septin genes *CDC3*, *CDC10*, *CDC11*, and *CDC12* commonly form branched, highly elongated, multinucleate cellular clusters (23, 31, 101). These mutants are unable to organize the bud neck filaments, which have essential roles in cytokinesis (23, 31, 101). Interestingly, the *S. cerevisiae* septins Cdc3, Cdc11, and Shs1/Sep7 contain sumoylation motifs. When these motifs were mutated, septin ring formation was lost, and there was also a loss of bud neck-associated SUMO (66). Septin sumoylation is dependent on the E3 enzyme Siz1 (67). Similar mechanisms may also operate in *C. albicans*. Although attempts to identify septin sumoylation have proved unsuccessful to date (105), *C. albicans cdc11* mutants display cell cycle defects similar to those of *smt3* mutants (174). Furthermore, *C. albicans* Cdc11 contains three type I (Ψ-K-X-E) consensus sumoylation sites, and Cdc3 contains two type I and one type II consensus sumoylation sites (141). To our knowledge, no one has tested the effects of mutating all sumoylation sites in Cdc11, although sumoylation appears to regulate a number of cell cycle-related proteins, and hence a number of cell cycle pathways. For example, Tub1, Cct7, and Mlc1 have been identified as sumoylation targets, suggesting that this posttranslational modification modulates tubulin and actin assembly, which could also account for the elongated, multinucleate cell phenotype of *C. albicans smt3* cells (87). It is conceivable that sumoylation of other bud neck proteins is required for normal morphogenesis.

C. albicans smt3 mutants also display susceptibility to a wide range of stresses, including temperature, oxidative, and cell wall stresses (87). Given the severe growth defects of these mutants, this was not surprising. Nevertheless, a number of *S. cerevisiae* proteins are sumoylated in response to stress (189), although the exact mechanisms by which sumoylation contributes to stress adaptation and/or survival are largely unknown. Similarly, the chaperones Hsp104 and Hsp60 were identified in a proteomics screen for stress-dependent sumoylation targets in *C. albicans*. Mutation of the consensus sumoylation site in Hsp104 caused cells to become more susceptible to thermal insults (87). Therefore, sumoylation of Hsp104 is important for thermal resistance, possibly by modulating Hsp104 activity.

An interesting phenotype was observed when the consensus sumoylation site in the mitochondrial chaperone Hsp60 was mu-

tated. A significant proportion of these cells grew in an elongated, pseudohypha-like fashion (87), suggesting a link between mitochondrial activity and filamentous growth in *C. albicans*. These $HSP60_{K324R}$ cells were also temperature sensitive, but only when cells were stressed with a respiratory inhibitor (87). Additional sumoylation targets may contribute to thermal resistance as a number of other *C. albicans* proteins appear to be sumoylated in response to stress (87). Indeed, sumoylation of HSF1 in mammalian cells is strongly induced by heat shock, which results in increased DNA binding and activity (59). Interestingly, HsfA2 sumoylation in *Arabidopsis* represses its transcriptional activity, suggesting that sumoylation can also negatively regulate proteins in response to stress (21).

Evidently, sumoylation contributes significantly to the control of growth, cell division, and stress adaptation, all of which are vital for fungal virulence (Fig. 1). Yet sumoylation is an understudied topic, especially in fungal pathogens. The mechanisms by which the sumoylation of specific target proteins control these cellular processes and hence fungal virulence remain to be elucidated.

Neddylation. Neddylation involves the covalent addition of the NEDD8 polypeptide (Rub1 in *S. cerevisiae*) to a small number of target proteins via an isopeptide linkage between the carboxy-terminal glycine (Gly)76 of NEDD8 and a lysine on the target protein (138). The mechanisms of protein neddylation are closely related to those involved in ubiquitination. The first identified target of Rub1 in *S. cerevisiae* was Cdc53 (84), which is a member of the cullin family of proteins (molecular scaffolds for ubiquitin ligases). Right across the eukaryotic kingdom from yeasts to humans, the cullin family has three to six members, suggesting the importance of neddylation in regulating cullin function. Indeed, all yeast cullins, including *S. cerevisiae* Cul3, Cdc53, and Rtt101 (85) and *S. pombe*: Pcu1, Pcu3, and Pcu4 (127, 187), are neddylated on a conserved lysine in their carboxy-terminal domain *in vivo*. This neddylation positively regulates E3 ligase activity and the assembly of Skp1/cullin/F-box (SCF) complexes (24, 84, 98). Defective neddylation causes severe defects in cell growth and development, ranging from cell cycle arrest in *S. pombe* to arrest in embryonic development for *Caenorhabditis elegans* (69).

Neddylation also contributes to fungal pathogenicity. *C. neoformans rub1* mutants exhibit severe survival defects when grown in cerebral spinal fluid, as well as attenuated virulence in *C. elegans* and a rabbit model of cryptococcal meningitis (89). Neddylation appears to be important for facilitating the attachment of ubiquitin E2 to the E3 ligase, allowing for efficient proteasome targeting of proteins (73). Therefore, this severe virulence defect could be due to defective protein turnover and adaptation to potentially host environments. A *RUB1* orthologue has been identified in *C. albicans*. Functional analysis of this gene is likely to confirm the importance of neddylation for fungal pathogenicity.

CONCLUDING REMARKS

A diverse range of posttranslational modifications regulate the activity and abundance of specific proteins in fungal pathogens, thereby controlling key cellular processes that contribute to the virulence of these organisms. While protein phosphorylation has received considerable attention through studies of signal transduction and transcriptional regulation, other functionally important posttranslational modifications (such as ubiquitination, sumoylation, and neddylation) have been largely ignored. It is evident that these processes contribute significantly to the growth and adaptation of fungal pathogens. The extent to which there is cross talk between posttranslational modifications is not known, but precedents clearly exist. For example, the phosphorylation of some target proteins is known to be a prerequisite for ubiquitination and subsequent substrate degradation (19). Although this topic has merely been touched upon in this review, it is clearly of high relevance and warrants further study. Therefore, further efforts are required to define the mechanisms by which these posttranslational modifications contribute to disease establishment and progression.

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